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DIFFERENTIATION OF *CLARIAS BATRACHUS*, *C. GARIEPINUS* AND *HETEROPNEUSTES FOSSILIS* BY PCR-SEQUENCING OF MITOCHONDRIAL 16S rRNA GENE

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Abstract

Heteropneustes fossilis, *Clarias batrachus* and *C. gariepinus* are three major catfishes of ecological and economic importance. Identification of these fish species becomes a problem when the usual external morphological features of the fish are lost or removed, such as in canned fish. Also, newly hatched fish larva is often difficult to identify. PCR-sequencing provides accurate alternative means of identification of individuals at species level. So, 16S rRNA genes of three locally collected catfishes were sequenced after PCR amplification and compared with the same gene sequences available from other geographical regions. Multiple sequence alignment of the 16S rRNA gene fragments of the catfish species has revealed polymorphic sites which can be used to differentiate these three species from one another and will provide valuable insight in choosing appropriate restriction enzymes for PCR-RFLP based identification in future.

Key words: PCR-sequencing, 16S rRNA, *Heteropneustes*, *Clarias*, Species identification

Introduction

Catfishes of the genus *Heteropneustes* and *Clarias* (Siluriformes: Heteropneustidae, Clariidae) are commercially and ecologically important groups of freshwater fishes. These closely related species share more or less conservative external morphology and are difficult to identify when usual external features are missing. For instance, during fish processing into portions of flesh, such as in canned fish, species identification could be impossible due to loss of identifying characteristics (Quinteiro *et al*. 1998). Also, newly hatched fish larvae are often difficult to differentiate based on morphology and alternative method is in use (Lindstrom 1999). In addition, species complexes are difficult to identify based on morphology alone.

Molecular techniques, especially DNA-based species identification techniques are better alternatives to morphology-based ones (Teletchea 2009). Analysis of polymerase chain reaction (PCR) amplified DNA fragments can provide an accurate alternative means of identification of individuals to genes or species level (Hubalkova *et al.* 2008, Jans *et al.*

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2012, Kochzius *et al.* 2010, Lakra *et al.* 2011, Scheidegger *et al.* 2009 and Teletchea 2009). Among many PCR-based approaches, PCR-sequencing is found to provide highest amount of information and has been widely used now-a-days (Hajibabaei *et al.* 2007, Rehbein 2013, Zeng *et al.* 2013 and Zhuang *et al.* 2013). PCR-RFLP (Restriction Fragment Length Polymorphism) is another good choice for differentiation of species with known sequence. PCR-RFLP of different genes was demonstrated to detect inter- and intra-specific variations in several animals (Di Finizio *et al.* 2007 and Duduk *et al.* 2013). PCR-RFLP analysis is faster and more cost effective.

Mitochondrial DNA (mtDNA) is maternally inherited and does not go through recombination event. Single nucleotide polymorphism in the mitochondrial genome increase the power of discrimination among the individuals (Coble *et al.* 2004). mtDNA genetic markers, such as Cytochrome oxidase I (COI), 16S and 12S rRNA genes have been widely used as tools to distinguish within and among species (Di Finizio *et al.* 2007, Klossa *et al.* 2002 and Wolf *et al.* 2000). Recently, 16S rRNA gene sequences are reported to be particularly useful in identifying different animal species (Sarri *et al.* 2014).

In this background, the main objectives of the present investigations are (1) to compare the 16S rRNA gene sequences of three catfishes from Bangladesh with the existing sequences of those in the GenBank database, (2) to compare sequences among the three selected catfishes and (3) to explore the possibility of identifying these three species using PCR-RFLP on 16S rRNA gene sequence.

Materials and Methods

Sample Collection and DNA Extraction: *H. fossilis* (locally known as Shing), *C. batrachus* (Magur) and *C. gariepinus* (African Magur) were collected from local fish markets (Dhaka, Bangladesh). DNA was extracted from 50 mg muscle tissue of each fish species following a standard method (Doyle and Doyle 1990) with modifications. CTAB extraction buffer (1M 10 ml Tris-HCl [pH 8], 0.5M 4ml Sodium-EDTA [pH 8], 8.18 g NaCl, 2g CTAB, dH_2O) and 10 µl of 20 mg/mL proteinase K were added with minced meat sample. The mixture was incubated at 55°C for 2 h. After digestion, the samples were centrifuged at 13000 rpm for 5 min and equal volume of Phenol: Chloroform was added to the supernatant. Then, DNA was precipitated with ethanol and dissolved in distilled water.

PCR amplification and Sequencing: Universal primers for PCR of the 16S rRNA gene, 16Sar-L (5'-CCGGTCTGAAAAAAACAT) and 16Sar-H (5'-CCGGTCTGAA CTCAG ATCACGT) have been utilized (Palumbi 1996). Standard PCR was performed using PCR Master Mix (Promega). Temperature regimes for PCR amplifications were as follows – initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30s and 72°C on the last cycle. PCR products were run in an agarose gel containing ethidium bromide. The electrophoresis was performed at 90 V for 30 min and

DNA bands were visualized on a UV transilluminator and photographed. Then, PCR amplicons were purified using a purification kit (FavorGen) and sequenced.

Bioinformatics: 16S rRNA gene sequences of experimental fish species and existing sequences of related fish species were collected from GenBank database (NCBI). Suitable portion of each sequence was taken for further observation. A multiple sequence alignment was performed using ClustalW software to observe the polymorphic sites present among the sequences. Restriction endo-nuclease cutting sites were searched in the sequences of the 16S rRNA fragment analyzed further. 'Serial Cloner' software was used to observe restriction sites.

Results and Discussion

Comparison of 16S rRNA gene sequence of three catfishes from Bangladesh with the available sequences from GenBank database: Partial sequences of 16S rRNA genes (5 *Clarias* spp. and 2 *Heteropneustes spp*.) were retrieved from GenBank database (Table 1). Suitable portion of DNA sequence (560 bp) was analyzed further. A multiple sequence alignment was done by SeaView to compare the sequences (Gouy *et al.* 2010) (Fig. 1). After comparing these sequences in different individuals, 55 out of 560 nucleotide bases of the sequence were found polymorphic. Among these polymorphic sites, nine were selected as positions with diagnostic value at genus level (Table 2). In seven positions (137, 219, 325, 367, 439, 443, 463) genus *Heteropneustes* and genus *Clarias* show nucleotide variations. In another two positions (242, 328), *Clarias* has particular nucleotide bases though deletion was observed in *Heteropneustes*. These diagnostic positions were selected using the criteria that these positions did not show intra-specific variability. In some positions, inter-species variations were also observed. 16S rRNA gene sequence of *H. fossilis* differs from that of *H. microps* in positions 34, 181, 241, 261, 313, 326, 331, 336, 338, 351, 361. Besides, *H. fossilis* sequence from Bangladesh differs from that of other geographical regions in the positions 271 and 377. Mitochondrial DNA diversity due to geographical differences has been reported elsewhere (Linares *et al.* 2009).

Table 1. List of 16S rRNA sequences used in this study.

Scientific name	Common name	GenBank Accession	Collected
Clarias batrachus	Walking Catfish (Magur)	KF997532.1*	Bangladesh
C. batrachus	Walking Catfish	JQ699193	India
C. gariepinus	North African Catfish (African	KJ819942*	Bangladesh
C. gariepinus	North African Catfish	JQ699188	India
C. dussumieri	-	JQ699198.1	India
C. fuscus	White spotted Clarias	JN020056.1	China
C. gabonensis	-	JX899749.1	Gabon
Heteropneustes	Stinging Catfish (Shing)	KJ819943*	Bangladesh
H. fossilis	Stinging Catfish	FN677932	India
H. fossilis	Stinging Catfish	GQ411079	India
H. microps	Stinging Catfish	FJ432686	India

*Sequences submitted to GenBank from this study. *C. dussumieri*

Sequence comparison among three selected catfishes: Fragments of mtDNA of three selected catfishes (*Clarias batrachus, C. gariepinus* and *Heteropneustes fossilis*) were sequenced. The GenBank accession numbers are KF997532.1, KJ819942 and KJ819943**,** respectively**.** A multiple sequence alignment was performed by SeaView (Gouy *et al*. 2010). Twenty nine out of 560 bases of the sequence were found polymorphic. Among the polymorphic sites, 15 have diagnostic value at genus level, i.e, genus *Clarias* and genus *Heteropneustes* can be differentiated by these 15 nucleotide variations (Table 3). Comparing the sequences, polymorphic sites were also observed between two species belonging to the genus *Clarias*. Fifteen positions were found which have diagnostic value at species level (Table 4). Two species of *Clarias* (*C. batrachus* and *C. gariepinus*) can be differentiated by this nucleotide variation.

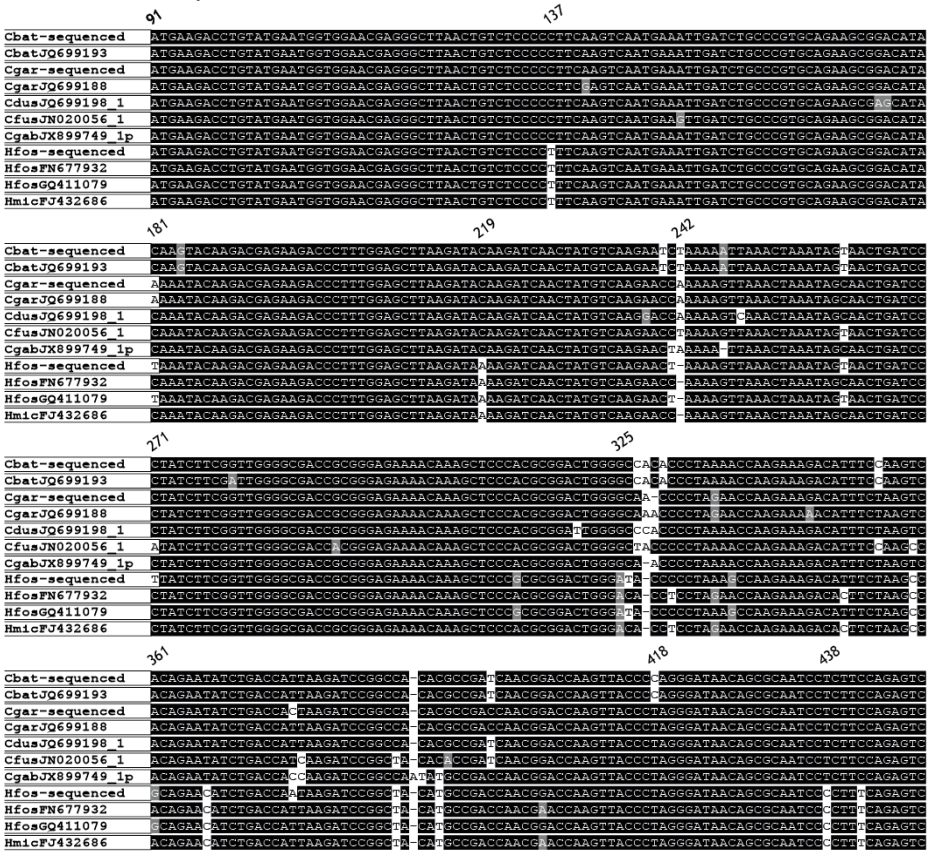

Fig. 1. Multiple sequence alignment of 16S rRNA gene fragments of *Clarias* spp. and *Heteropneustes* spp. Some representative polymorphic sites are indicated by the nucleotide position numbers of *Clarias batrachus* (Cbat) sequence on the top.

Table 2. Polymorphic sites observed between two catfish genus from Bangladesh with the existing sequences of those in the GenBank database in 16S rRNA gene fragment with diagnostic value.

Position	*Heteropneustes* spp.	*Clarias* spp.
137	T	C
219	A	C
242	nucleotide deleted	T or A
325	A	G
328	nucleotide deleted	A or C
367	C	T
439	C	T
443	T	C
463	G	A

Table 3. Polymorphic sites observed among three native catfishes from Bangladesh in 16S rRNA gene fragment with diagnostic value (differentiation at genus level).

Position	*Clarias spp.*	*Heteropneustes spp.*
137	C	T
219	C	A
271	C	T
313	A	G
325	G	A
338	C	T
359	A	G
361	T	C
367	A	G
389	C	T
393	C	T
438	T	C
442	C	T
462	A	G

Possible differentiation of three species using PCR-RFLP on *16S rRNA* gene: Partial 16S rRNA sequences of the three catfishes were tested with various restriction enzymes using open source molecular biology software Serial Cloner (http://serialbasics.free.fr/Serial_Cloner.html). Some of the enzymes were found which are able to differentiate the selected species. This bioinformatic analysis has identified three restriction enzymes that are useful for distinguishing among the catfishes (Table 5). Restriction enzymes for RFLP analysis were selected based on two criteria. Firstly, it has the ability to provide differentiation among the species and secondly, it produces fragments of suitable sizes easy to visualize in agarose gel. PCR-RFLP using these three restriction enzymes, *AfaI*, *NspI* and *PasI* can separate two genus *Heteropneustes* and *Clarias*, although there are some other enzyme options as well. Besides, two species of the same genus *Clarias* (*C. batrachus* and *C. gariepinus*) can also be differentiated by these enzymes. So, the restriction enzymes *AfaI*, *NspI* and *PasI* are candidates of highest diagnostic value for the selected three species. Once experimental proof is obtained, PCR-RFLP using these three enzymes will help for easy and cheaper differentiation of the selected catfishes.

Table 4. Polymorphic sites observed between two *Clarias* species in 16S rRNA gene fragment with diagnostic value (differentiation at species level).

Position	*C. batrachus*	*C. gariepinus*
181	C	A
184	G	A
240	T	C
242	T	A
247	A	G
261	T	C
327	C	A
330	A	deletion
336	A	G
355	C	T
377	T	C
399	T	C
418	C	T
560	C	T

Table 5. Restriction enzymes (RE) which allow differentiation among three species.

RE with cutting sites	Position	*C. batrachus*	*C. gariepinus*	*H. fossilis*
AfaI GTAC CATG	184	G (digested)	A (not digested)	A (not digested)
NspI RCATGY YGTACR	389	C (not digested)	C (not digested)	T (digested)
PasI CCCWGGG GGGWCCC	418	C (digested)	T (not digested)	T (not digested)

Both mitochondrial and nuclear gene sequences are used for the purpose of molecular species identification (Rehbein 2013 and Hajibabaei *et al.* 2007). Mitochondrial genes, such as, Cytochrome oxidase I (COI), 16S and 12S rRNA genes are commonly used markers for molecular species identification (Cawthorn *et al.* 2012 Di Finizio *et al.* 2007 and Zhuang *et al.* 2013). Among the three mitochondrial genes, 16S rRNA gene has been more suitable because it is highly conserved among the vertebrates and has lower sequence variation among the populations of same species (Kitano *et al.* 2007, Di Finizio *et al.* 2007, Cawthorn *et al.* 2012 and Kochzius *et al.* 2010). Compared to *COI*, 16S rRNA gene has been reported to have lower K2P distances (about 10 fold) within same species (Cawthorn *et al.* 2012 and Lakra *et al.* 2011). In a study of eight pecoran species, 16S rRNA gene was found to have a larger number of species-specific polymorphic sites compared to the cytochrome b gene indicating the usefulness of 16S rRNA gene for species identification (Guha *et al.* 2006). In this circumstance, analyzing partial sequences of 16S rRNA gene for molecular differentiation of three selected catfishes of Bangladesh is a timely work, if not late. The results also prove that how this work can help to differentiate these three species. Moreover, using appropriate restriction enzymes

(as indicated by this study), these three species can be separated by RFLP as well, which will be a less time-consuming and cheaper practice.

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